

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

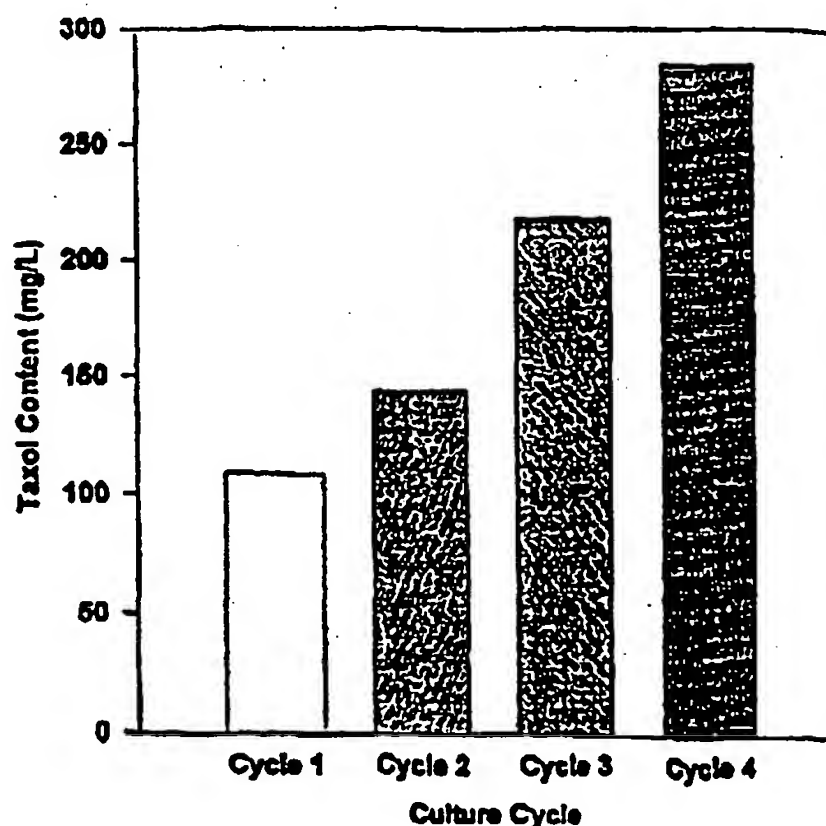
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12P 17/02		(11) International Publication Number: WO 96/34110
A1		(43) International Publication Date: 31 October 1996 (31.10.96)
(21) International Application Number: PCT/KR96/00060		Narae Apartment, 462-4, Jeonmin-dong, Yusong-ku, Taejon 305-390 (KR). HONG, Seung-Suh [KR/KR]; 109-404, Cheonggu-Narae Apartment, 462-4, Jeonmin-dong, Yusong-ku, Taejon 305-390 (KR). LEE, Hyun-Soo [KR/KR]; 1331-603, Mokdong-Sinsigaji Apartment, 328, Sinjung-dong, Yangchun-ku, Seoul 158-070 (KR). (74) Agent: LEE, Han-Young; 301, Yuyang Building, 823-11, Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR). (81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 27 April 1996 (27.04.96)		
(30) Priority Data: 1995/10204 27 April 1995 (27.04.95) KR		
(71) Applicant (for all designated States except US): SAMYANG GENEX CO., LTD. [KR/KR]; 263, Yonji-dong, Chongno-ku, Seoul 110-470 (KR).		
(72) Inventors; and (75) Inventors/Applicants (for US only): CHOI, Hyung-Kyoon [KR/KR]; 103-809, Sangroksu Apartment, 281, Mamyun-dong, Seo-ku, Taejon 302-150 (KR). ADAMS, Tom, Lee [US/US]; 346 Richmond Drive #4, Millbrae, CA 94030 (US). STAHLHUT, Roy, William [US/US]; 2320 Coronet Boulevard, Belmont, CA 94002 (US). KIM, Sang-ic [KR/KR]; 102-605, Daerim-Byuksan Apartment, Chunggye-dong, Nowon-ku, Seoul 139-220 (KR). YUN, Jeong-Hwan [KR/KR]; 63-2, Hwaam-dong, Yusong-ku, Taejon 305-348 (KR). SONG, Bong-Kyu [KR/KR]; 109-303, Cheonggu-Narae Apartment, 462-4, Jeonmin-dong, Yusong-ku, Taejon 305-390 (KR). KIM, Jin-Hyun [KR/KR]; 3-303, Soojung Apartment, Samchun-dong, Seo-ku, Taejon 302-222 (KR). SONG, Jun-Seog [KR/KR]; 104-305, Cheonggu-		
Published With international search report. With an indication in relation to a deposited microorganism furnished under Rule 13 ^{bis} separately from the description. Date of receipt by the International Bureau: 21 May 1996 (21.05.96)		

(54) Title: A METHOD FOR MASS PRODUCTION OF TAXOL BY SEMI-CONTINUOUS CULTURE

(57) Abstract

The present invention relates to a method for mass production of taxol by semi-continuous culture of *Taxus* genus plant with a high yield. According to the present invention, taxol can be prepared with a high yield, by employing semi-continuous culture of *Taxus* genus plant cell, which comprises: (i) inoculating *Taxus* genus plant cell on a medium containing 1 to 10 % (w/v) sugar, and incubating it; and, (ii) transferring 1/10 to 1/2 volume of the culture obtained in the step (i) to a fresh medium and repeating the step (i), adding 1 to 10 % (w/v) sugar to the remnant culture and incubating to the time of maximum production of taxol.



BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AN	Armenia	GB	United Kingdom	MT	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Switzerland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TO	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

A METHOD FOR MASS PRODUCTION OF TAXOL
BY SEMI-CONTINUOUS CULTURE

BACKGROUND OF THE INVENTION

5

Field of the Invention

The present invention relates to a method for mass production of taxol from TAXUS genus plant cell culture, more specifically, to a method for mass production of taxol by semi-continuous culture of TAXUS genus plant cell with a high yield.

Description of the Prior Art

15

Taxanes are diterpene compounds containing a taxane skeleton. For example, taxol is famous as the first identified compound with a taxane ring which was isolated from the bark of the pacific yew, TAXUS brevifolia, which is effective for the treatment of leukemia and cancer. Recently, it has been reported that: taxol is capable of curing approximately 30%, 50% and 20% of ovarian, breast and lung cancer patients, respectively, by inhibiting depolymerization of microtubules (see: E.K. Rowinsky et al., J. Natl. Cancer. Inst., 82:1247-1259(1990)).

On the other hand, total chemical synthesis, semi-synthesis and extraction methods have been employed to prepare taxol.

The total chemical synthesis method, however, has not been practically applied in the art, since it requires very expensive chemical reagents and the yield is not so high, which can be expected from the complicated chemical structure of taxol.

The semi-synthesis method employing precursors such as 10-deacetylbaccatin III, has revealed some drawbacks since it essentially entails complicated and multiple steps of isolating and purifying the taxol precursors from TAXUS

genus plant and transforming the precursors to taxol.

In this regard, the extraction method by which taxol can be isolated from Taxus genus plants in a direct manner, has prevailed in the art, since it has the advantage of economy. However, the said method has proven to be less satisfactory in the sense that it essentially requires a large amount of yew trees to purify taxol, which finally gives rise to the serious environmental disruption.

Accordingly, the ability of total chemical synthesis, semi-synthesis and extraction method to supply taxol for world-wide chemotherapeutic use is not assured; and, there are strong reasons for exploring and developing alternative means of taxol production.

As a promising alternative to solve said problems, the cell culture method for taxol production has been proposed in the art.

The cell culture-based process for taxol production, unlike the prior art, has the following advantages as followings: first, taxol can be produced in a steady manner, regardless of fluctuation of supply of yew plants due to the damage by blight and harmful insects, etc.; secondly, cell cultures can be propagated in large bioreactors, from which taxol can be massively produced by manipulating culture conditions; thirdly, cell cultures produce a simpler spectrum of compounds compared to other prior art methods, considerably simplifying separation and purification; fourthly, a cell culture process can adapt quickly to rapid changes in demand better than the other methods; fifthly, a cell culture process can produce taxol as well as taxane precursors such as baccatin that can be converted to taxol.

Methods for producing taxol by utilizing cultured plant cells have been described in the art:

USP 5,019,504 discloses a method for producing taxol and its derivative utilizing cultured cells of Taxus brevifolia. However, the yield of taxol described therein is 1-3 mg/L, which is insufficient for industrial

application. Besides, the production of taxol by the cell culture is unstable and even when a primary cell of high productivity can be obtained by selection, it is difficult to keep its content by subculturing(see: E. R. M. Wickremesine et al., World Congress on Cell and Tissue Culture(1992)).

USP 5,015,744 teaches a semi-synthetic method from baccatin III, which is a precursor in biosynthesis of taxol. By the use of the plant tissue culture, a raw material for the semi-synthetic process such as baccatin III can be produced, thus the plant tissue culture can also be utilized for taxol production by the above-mentioned semi-synthetic process.

WO 93/17121 offers a method for taxol production by cell culture of Taxus genus plant while changing composition of medium, growth rate, and production rate, etc. In case of Taxus chinensis, 24.1mg/L of taxol can be obtained in 18 days of culture and biomass doubles every 2.5 days.

All of these patents describes methods for mass production of taxol by employing batch culture; there is no teaching in said patents on, nor is there anticipated, semi-continuous culture of a taxol-producing cell line.

Under the circumstances, USP 5,407,816 describes that Taxus chinensis cells are inoculated to a nutrient medium to form a suspension which is, in turn, cultivated to form a suspension culture which is, in turn, subcultured in the other nutrient medium to form a producing culture, and which finally gives taxol and taxanes in a yield of 153 mg/L. The said method has considerably improved the productivity of taxol, however, it has been proven less satisfactory in the sense that it essentially requires so many nutrient media whose compositions are so complicated, and high productivity can be realized under rather a limited growth condition.

Therefore, there is a continued need to develop a practical and simple method for taxol production, which is

able to meet the requirement of high productivity which is a critical factor to determine whether it can be utilized in industrial applications or not.

5 SUMMARY OF THE INVENTION

In accordance with the present invention, it has been discovered that taxol can be produced with a high degree of efficiency, by the semi-continuous culture of a novel
10 taxol-producing cell line, Taxus chinensis SYG-1.

A primary object of the present invention is, therefore, to provide a method for mass production of taxol by semi-continuous culture.

15 The other object of the invention is to provide a novel taxol-producing cell line isolated from Taxus chinensis.

BRIEF DESCRIPTION OF THE DRAWINGS

20

The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

25

Figure 1 is a graph showing the effect of AgNO_3 on the taxol productivity.

30

Figure 2 is a graph showing the effect of NH_4 -citrate on the taxol productivity.

Figure 3 is a graph showing the effect of maltose on the taxol productivity.

35

Figure 4 is a graph showing the productivity of taxol in semi-continuous culture of SYG-1 in accordance with the culture cycle.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors first developed a taxol-producing cell line, based on the callus induced from Taxus chinensis, and compared the cell line with those known in the art. From the comparative studies on the morphological, physiological and growth condition of the taxol-producing cell lines, it has been determined that the newly developed cell line is a novel one which is somewhat different from those of prior art, in light of taxol productivity, mode of taxol secretion, etc. Accordingly, the cell line was named Taxus chinensis SYG-1(hereinafter, referred to as "SYG-1" for convenience) and it was deposited with an International Depositary Authority(IDA), the Korean collection for Type Cultures(KCTC) on March 14, 1996, under an accession number of KCTC 0232BP.

Then, the inventors optimized the growth condition of the cell line of the invention, and determined that: SYG-1 cells grown well on B5 medium, most preferably B5 medium supplemented with 20 μ M NAA(naphthoxyacetic acid), 0.4 μ M BAP(6-benzylaminopurine), 1g/L casein hydrolysate and 30g/L sucrose, under a condition of a temperature of 24°C, at an agitation speed of 150rpm. On the other hand, effects of AgNO₃, NH₄-citrate and maltose on the productivity of taxol were also examined, and the inventors concluded that their addition considerably improved the productivity of taxol.

According to the present invention, taxol can be prepared with a high yield, by employing semi-continuous culture of SYG-1, which comprises:

- (i) inoculating Taxus genus plant cell on a medium containing 1 to 10%(w/v) sugar, and incubating it; and,
- (ii) transferring 1/10 to 1/2 volume of the culture obtained in the step (i) to a fresh medium and repeating the step (i), adding 1 to 10%(w/v) sugar to the remnant culture and incubating to the time of maximum production of

taxol.

At the beginning of culture, AgNO_3 may be added to Taxus genus plant cell culture which is incubated for 10 to 20 days, more preferably 10 to 15 days, at a concentration of 1 to 15 μM , more preferably 5 to 10 μM . Furthermore, NH_4 -citrate and maltose may be added to the culture after 5 to 30 days, more preferably 5 to 10 days of incubation, at a concentration of 1 to 15 mM , more preferably 1 to 10 mM and 1 to 10% (w/v), more preferably 1 to 5% (w/v), respectively.

After 5 to 30 days of incubation, 1/10 to 1/2 volume of total culture is transferred to other flask containing fresh medium whose contents are the same as the one employed at the beginning of the culture, and started a cycle of culture. Then, remnant culture equivalent to 9/10 to 1/2 volume of total culture, was added 1 to 10% (w/v), more preferably 1 to 5% (w/v) of maltose, and incubated for 30 to 60 days in which taxol production is maximized. At this time, incubation was made at 24°C, agitation speed of 150rpm under a dark condition.

Taxus genus plant employed in the method of the present invention includes Taxus brevifolia, Taxus canadensis, Taxus cuspidata, Taxus baccata, Taxus globosa, Taxus floridana, Taxus wallichiana, Taxus media and Taxus chinensis, however, Taxus chinensis SYG-1 which is newly developed by the inventors, is most preferably employed therein.

According to the method of present invention, the productivity of taxol to the level of 300mg/L, after 42 to 49 days incubation, is observed, when Taxus chinensis SYG-1 is employed in the semi-continuous culture with the addition of AgNO_3 , NH_4 -citrate and maltose to improve the productivity of taxol.

35 Quantitative Analysis of Taxol

Taxol which is produced from the culture of Taxus

chinensis SYG-1 according to the method of the present invention, is quantitatively assayed by employing high performance liquid chromatography under a specific condition described in Table 1 below.

5

Table 1: Condition for quantitative assay of taxol

	Instrument	HPLC(Waters, U.S.A.)
	Column	Capcell Pack C ₁₈ UG 120 (length: 250mm, inner diameter: 4.6mm)
10	Column temp.	40°C
	Mobile phase	CH ₃ CN : water(20 ~ 100% gradient)
	Fluid speed	1.0 ml/min
	Injection volume	10 µl
	Detector	UV(227nm), ATTE = 3

15

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

20 Example 1: Induction and characterization of callus from Taxus chinensis

The cell line of the present invention was isolated from Taxus chinensis by way of callus induction. When
25 plant tissue was incubated on a proper medium containing a hormone, undifferentiated cell of callus was induced: Tissues from bark, needles, stems and roots of Taxus chinensis were washed with tap water and sterilized with Ca-hypochloride solution for 20 to 30min. Then, the
30 sterilized tissue was washed with distilled water 2 to 3 times, chopped to have a length of 1cm, and transferred to B5 medium(see: Gamborg et al., Can. J. Biochem., 46:417-421(1968)) supplemented with 20µM NAA(naphthoxyacetic acid), 0.4µM BAP(6-benzylaminopurine), 1g/L casein

hydrolysate and 30g/L sucrose after solidifying with 0.2% (w/w) gelite, and incubated at a temperature of 24 to 26°C for 2 to 6 weeks under a dark condition to induce the callus of interest.

- 5 The growth medium for callus was selected by transferring the callus to several media well known in the art, e.g., MS(see: Murashige, T. and F. Skoog, F., Physiol. Plant, 5:473(1962)), SH(see: Schenk and Hilderbrandt, Can. J. Bot., 50:199-204(1972)), WPM(see: 10 Lloyd and McCown, Int. Plant Prop. Soc. Proc., 30:421-427(1981)) and B5 media(see: *supra*), respectively, and observing the growth pattern of the callus by naked eye. From the results above, it was determined that the callus showed good growth on B5 medium containing 20μM NAA, 0.4μM 15 BAP and 2g/L casein hydrolysate.

Then, studies on the morphology, physiology and growth condition were made for the cell line thus induced, and compared with those of known in the art; and, the results were summarized in Tables 2 and 3 below. As can be seen in 20 Tables 2 and 3, it was determined that the cell line which has been developed in the invention has a distinction over those of prior art, in light of taxol productivity and mode of taxol secretion, etc. Accordingly, the cell line was named Taxus chinensis SYG-1 and was deposited with an 25 International Depositary Authority(IDA), the Korean Collection for Type Cultures(KCTC) on March 14, 1996, under an accession number of KCTC 0232BP.

Table 2: Morphology and growth condition of Taxus chinensis
SYG-1

	<u>Taxus chinensis</u> SYG-1	Characteristics
5	Size	50-150 μ m
	Mobility	(-)
	Agglutination	Weak
	Aggregation	(+)
	Adaptation to shear stress	Strong
10	Color of culture	Weak brown
	Culture temperature	24°C

15

20

25

30

Example 2: Suspension culture of SYG-1

To select the most preferred medium for the suspension culture of SYG-1, the cell line was transferred to MS, SH, WPM and B5 media, respectively, and the growth pattern was observed analogously in Example 1, which resulted that SYG-1 cell was well grown on B5 medium containing 20 μ M NAA, 0.4 μ M BAP and 2g/L casein hydrolysate, like the callus. In addition, it was also determined that SYG-1 cell was well grown at a temperature of 24°C, at an agitation speed of 150rpm.

A serial cultivation of the callus was made every 4 weeks in a case of solidified medium, while transferring a part of tissue by the aid of forceps: A piece of the callus was maintained on the plate of solid medium. Then, the callus maintained on the solid medium was inoculated into a small volume of B5 medium, and small amounts of medium were supplemented to the culture, as the cells grew to increase total volume of culture. Then, well grown cell lines were diluted in the modified B5 medium contained in 500ml Erlenmeyer flask at a ratio of 1/5(v/v), and inoculated into suspension culture medium every two weeks.

Example 3: Effect of AgNO₃ on the taxol productivity

25

It has been well known that AgNO₃ is an antagonist of the plant hormone, ethylene, which effects on the plant cell growth and secondary metabolite production. Accordingly, the present inventors tested the effect of AgNO₃ on the taxol production in a suspension culture of SYG-1.

To a 250ml of Erlenmeyer flask, was poured 75ml of B5 medium containing 10 μ M AgNO₃ and 25ml of 14 day old cell culture was inoculated into this medium, and incubated in an analogous manner described in Example 2. Then, the productivity of taxol was compared with a control which does not contain AgNO₃ (see: Fig. 1). As can be seen in Fig.

1, it was clearly determined that the amount of taxol produced, 98.95mg/L when AgNO₃ was added to a medium(-O-), was 4.7 times the control(-O-).

5 Example 4: Effect of NH₄-citrate on the taxol productivity

The productivity of taxol in a suspension culture of SYG-1 was monitored, after the addition of NH₄-citrate. To a 250ml of Erlenmeyer flask was poured 75ml of B5 medium and 25ml of 14 day old cell culture was inoculated into this medium, and incubated at the same growth condition described in Example 2. Then, 5mM of NH₄-citrate was added to the culture after 9 days of incubation, and the taxol productivity was compared with a control in which NH₄-citrate was not added(see: Fig. 2). As can be seen in Fig 2, it was determined that the amount of taxol produced was about 103.6mg/L when NH₄-citrate was added to a medium(-O-), which was 4.9 times the control(-O-).

20 Example 5: Effect of maltose on the taxol productivity

It has been well known that the increase of sugar concentration in a plant cell culture leads to an increase in secondary metabolite production. For example, it has been reported that: addition of 3%(w/v) sucrose and 5%(w/v) mannitol has improved the productivity of antocyanin in callus culture of Daucus carota(see: Knobloch, K. -H., et al., Zeiteschrift fur Naturforschung, 35c:55-556(1981)); and, 88mM sucrose and 165mM mannitol heightened the antocyanin productivity in a suspension culture of Vitis vinifera(see: Rajendran, L., et al., Biotechnology Letters, 14(8):707-712(1992)). On the other hand, the decrease in the growth and the secondary metabolite production were also reported when the amount of sugar over a certain concentration was added, possibly due to osmotic stress(see: Do, C. B., and Cormier, F., Plant Cell Reports, 9:500-504(1990)).

The effect of maltose addition to SYG-1 was evaluated.

To a 250ml of Erlenmeyer flask was poured 75ml of B5 medium and 25ml of 14 day old cell culture was inoculated into this medium, and incubated under the same growth condition described in Example 2. Then, 2% (w/v) and 3% (w/v) of maltose were added to the culture after 9 days and 21 days of incubation, respectively and the productivity of taxol was compared with a control which does not contain maltose (see: Fig. 3). As can be seen in Fig. 3, it was determined that the amount of taxol was 112.75mg/L when maltose was added to a medium (-S-), which was 5.3 times the control (-O-).

Example 6: Semi-continuous culture of SYG-1

15

Based on the results obtained in Examples 2 to 5, taxol was prepared by employing semi-continuous culture of SYG-1.

To a 250ml of Erlenmeyer flask was added 80ml of growth medium and 20ml of 14 day old SYG-1 culture, 10 μ M AgNO₃ was added at the beginning of culture, followed by the addition of 5mM NH₄-citrate and 2% (w/v) maltose after 9 days of incubation. After 21 days of incubation, 20ml of culture equivalent to 1/5 volume of total culture was transferred to other flask containing 80ml fresh medium to start another cycle of culture. Then, to 80ml of remnant culture equivalent to 4/5 volume of total culture, was added 3% (w/v) maltose, and incubated for additional 21 days in which taxol production is maximized. At this time, incubation was made at 24°C, and an agitation speed of 150rpm. Culture samples were taken, periodically to check for microbial contamination. The amount of taxol produced in the culture was also determined by employing high performance liquid chromatography (HPLC, Waters, U.S.A.) described as above. As a result, microbial contamination was not observed and the productivity of taxol after 42 days incubation was determined to be 284mg/L (see: Fig. 4).

14

As can be seen in Fig. 4, taxol concentration was increased as the cycles of culture were repeated.

As clearly illustrated and demonstrated as above, the present invention provides a method for mass production of taxol by semi-continuous culture of Taxus genus plant with a high yield. According to the present invention, the productivity of taxol to the level of 300mg/L, after 40 to 50 days incubation, was observed, when Taxus chinensis SYG-1 was employed in the semi-continuous culture with the addition of AgNO_3 , NH_4 -citrate and maltose to improve the productivity of taxol.

15

20

25

30

35

WHAT IS CLAIMED IS:

1. A method for mass production of taxol by semi-continuous culture which comprises the steps of:

- 5
- (i) inoculating Taxus genus plant cell on a medium containing 1 to 10%(w/v) sugar, and incubating it; and,
 - 10 (ii) transferring 1/10 to 1/2 volume of the culture obtained in the step (i) to a fresh medium and repeating the step (i), adding 1 to 10%(w/v) sugar to the remnant culture and incubating to the time of maximum production of taxol.

15 2. The method of claim 1, wherein the Taxus genus plant is selected from the group consisting of Taxus brevifolia, Taxus canadensis, Taxus cuspidata, Taxus baccata, Taxus globosa, Taxus floridana, Taxus wallichiana, Taxus media and Taxus chinensis.

20 3. The method of claim 1, wherein the Taxus genus plant cell is Taxus chinensis SYG-1(KCTC 0232BP).

25 4. The method of claim 1, which further comprises a step of addition of AgNO_3 to the medium at a concentration of 1 to $15\mu\text{M}$ at the beginning of cell culture.

30 5. The method of claim 1, which further comprises a step of addition of NH_4 -citrate or maltose, or both, to the medium at a concentration of 1 to 15mM and 1 to 10%(w/v), after 5 to 30 days of incubation, respectively.

35 6. The method of claim 1, wherein the time of maximum production of taxol is 40 to 50 days from the beginning of cell culture.

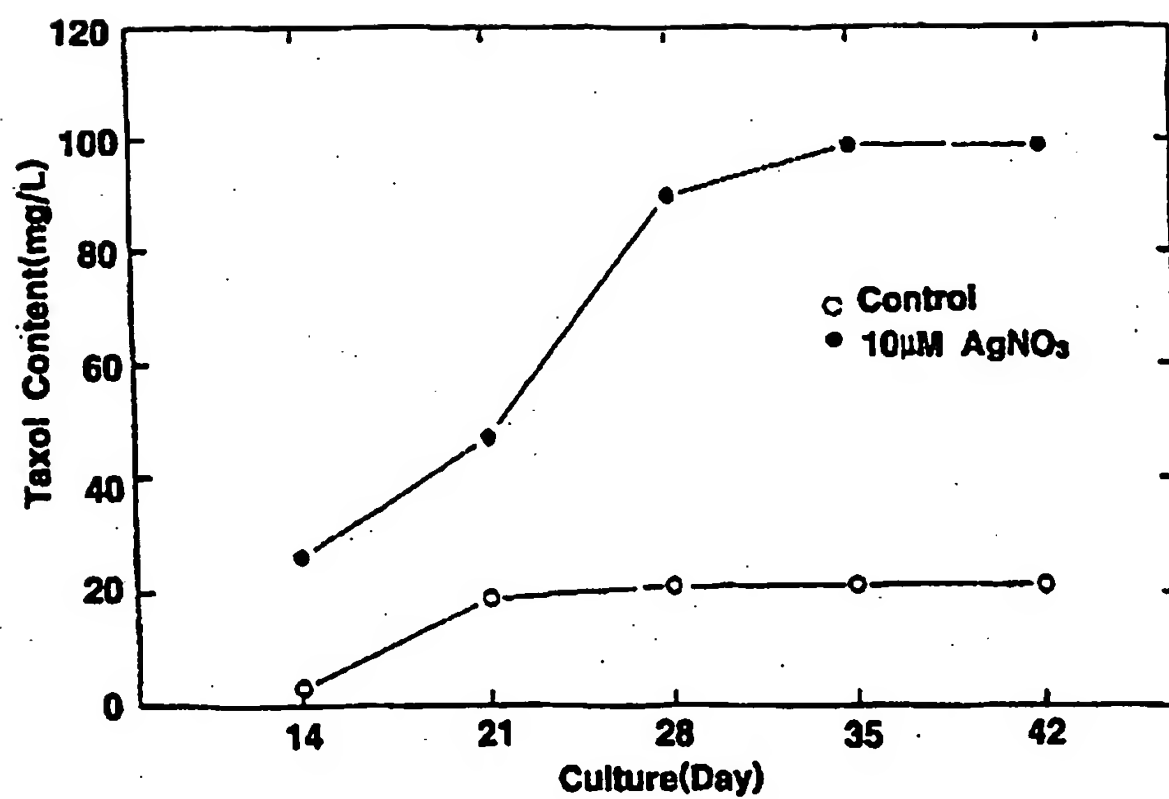


FIGURE 1

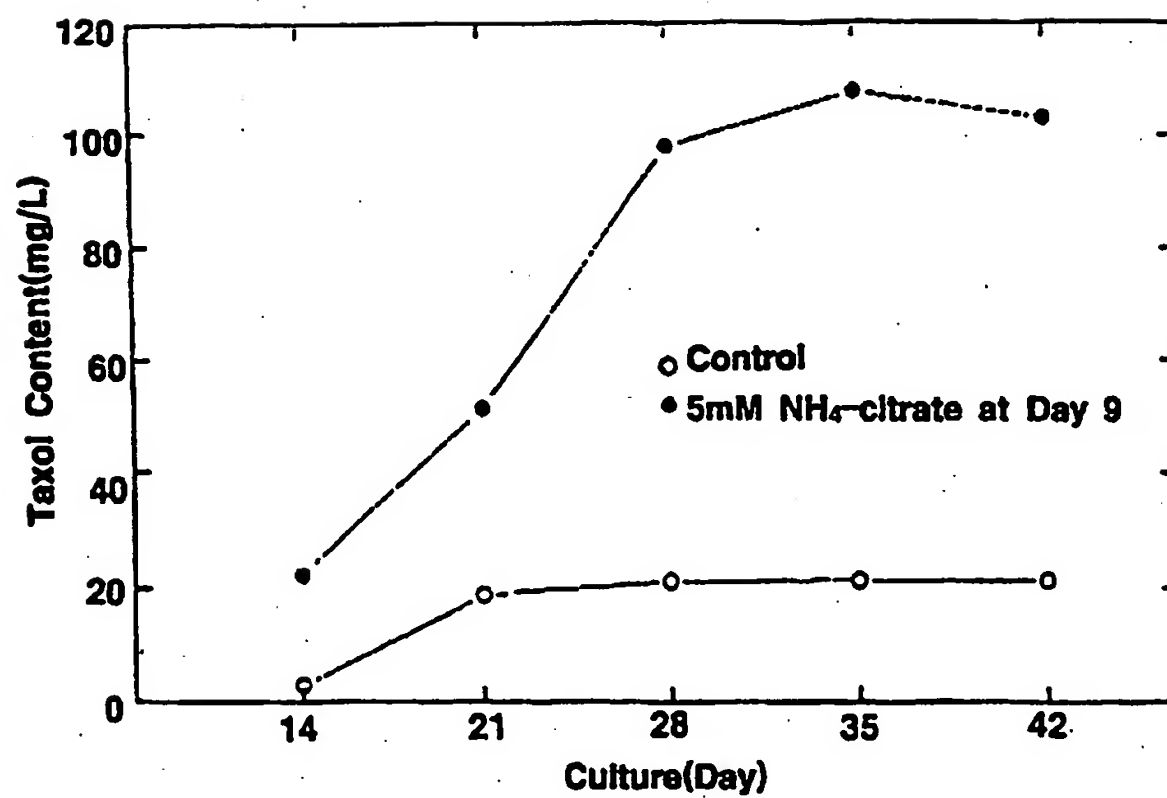


FIGURE 2

3/4

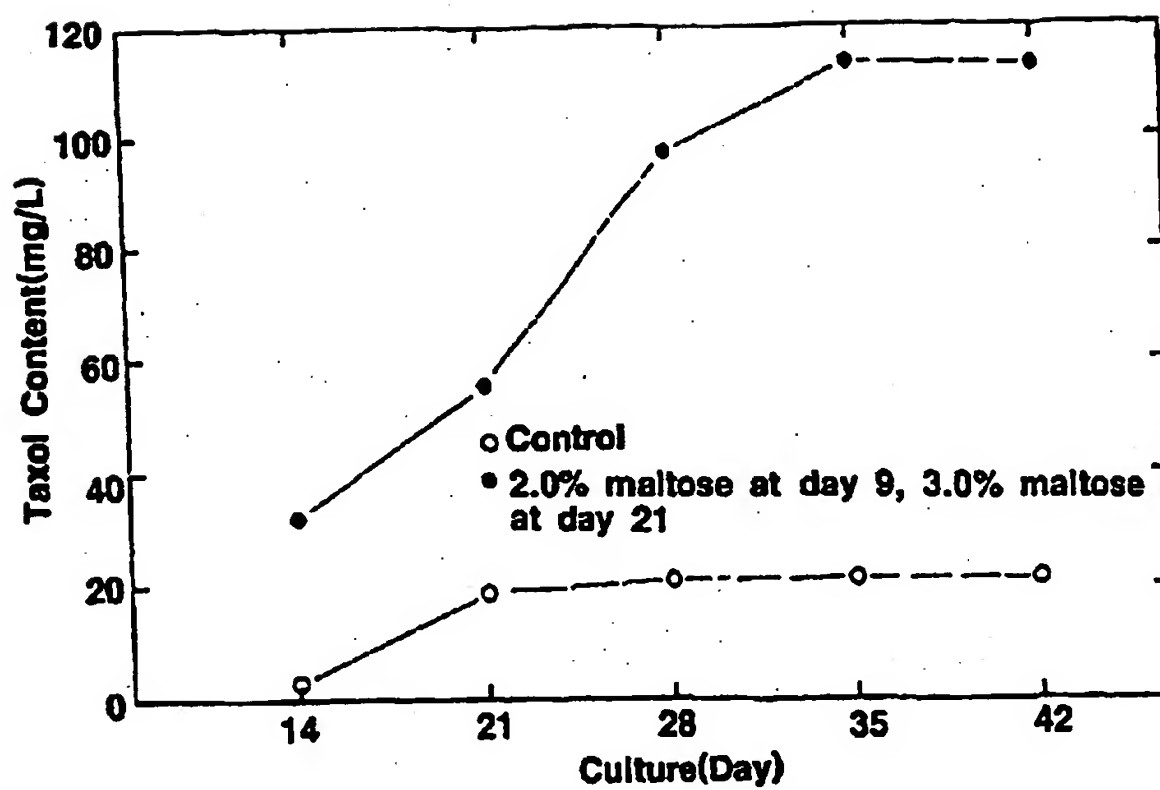


FIGURE 3

4/4

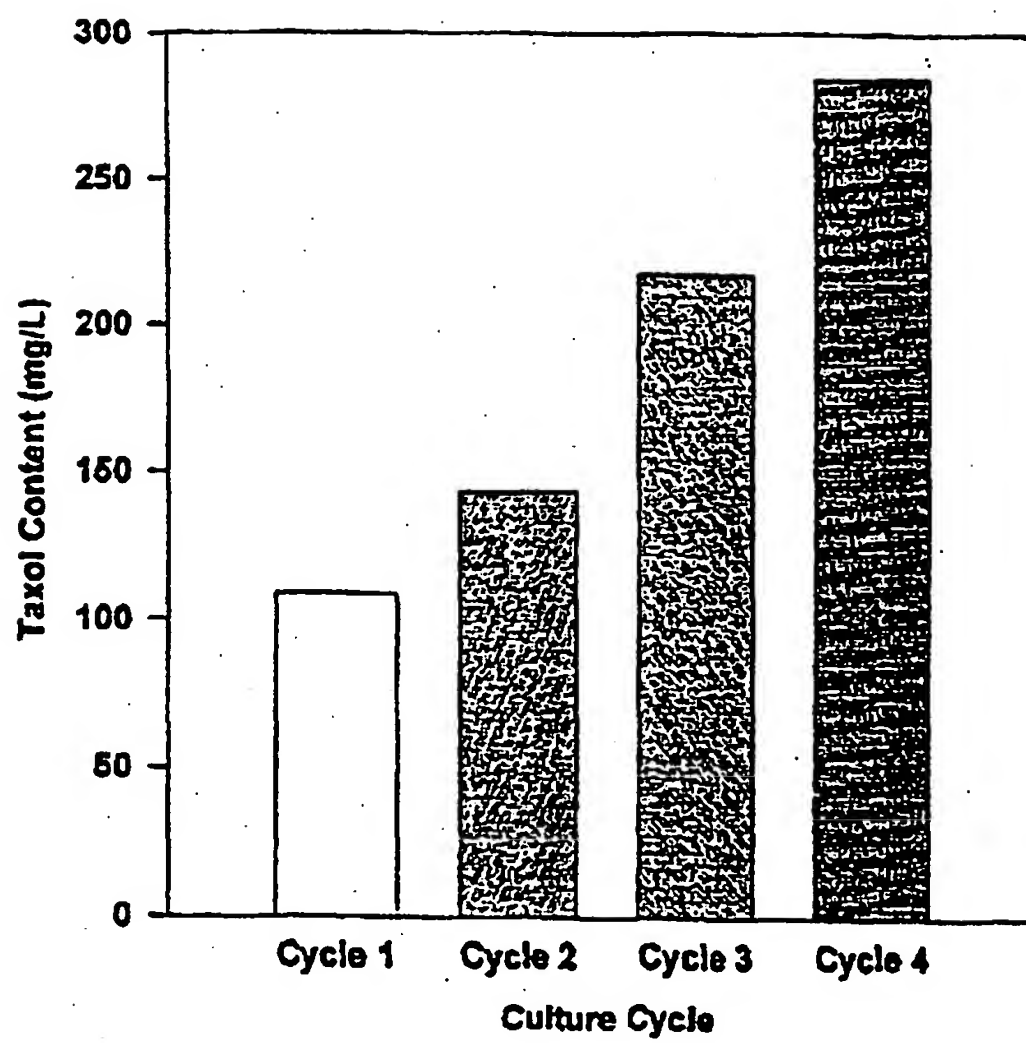


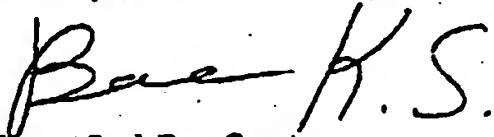
FIGURE 4

ECT/KR96/00060

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM
RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1

TO: Kim, Kyung Hwan
#263 Yonji-dong, Chongno-ku, Seoul 110-725,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Taxus chinensis SYG-1	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0232BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on March 14 1996.	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures Address: KCTC, KRIBB #52 Oin-dong, Yusong-ku, Taejeon 305-600, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Hyung Sook Bae, Curator Date: March 26 1996

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 96/00060

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: C 12 P 17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: C 12 P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/19 585 A1 (UNION CAMP CORPORATION) 14 October 1993 (14.10.93), claims 1,2,12,13; page 23.	1,2
A	WO 93/17 121 A1 (PHYTON CATALYTIC, INC.) 02 September 1993 (02.09.93), claims 1,9,21,22.	1-3
A	US 5 019 504 A (CHRISTEN et al.) 28 May 1991 (28.05.91), claims 1-4,9.	1

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "T" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to underpin the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"A" document member of the same patent family

Date of the actual completion of the international search

08 July 1996 (08.07.96)

Date of mailing of the international search report

18 July 1996 (18.07.96)

Name and mailing address of the ISA/AT
AUSTRIAN PATENT OFFICE
Kohlmarkt 8-10
A-1014 Vienna
Facsimile No. 1/53424/535

Authorized officer

Telephone No. Wolf
1/53424/133

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR 96/00060

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9319585	14-10-93	AU A1 39398/93 EP A1 633719 EP A4 633719 IL A0 105199 JP T2 7508163 US A 5310672	08-11-93 18-01-95 20-12-95 08-07-95 14-09-95 10-05-94
WO A1 9317121	02-09-93	AU A1 37291/93 EP A1 672162 EP A4 672162 JP T2 7506721 US A 5407816	13-09-93 20-09-95 29-11-95 27-07-95 18-04-95
US A 5019504	28-05-91	keine - none - rien	

MicroPatent® Family Lookup

Stage 1 Patent Family - "Complex"		Priorities and Applications	
CC DocNum	KD PubDate	CC AppNum	KD AppDate
<input type="checkbox"/> AT 195146	T 20000815	AT 93906156 US 9301576 US 83914492 US 87434492	T 19930222 W 19930222 A 19920220 A 19920424
<input type="checkbox"/> AT 256197	T 20031215	AT 99202507 US 87434492 US 83914492	T 19930222 A 19920424 A 19920220
<input type="checkbox"/> AU 3729193	A 19930913	AU 3729193 US 9301576 US 83914492 US 87434492	A 19930222 W 19930222 A 19920220 A 19920424
<input type="checkbox"/> AU 672194	B2 19960926	AU 3729193 US 9301576 US 83914492 US 87434492	A 19930222 W 19930222 A 19920220 A 19920424
<input type="checkbox"/> CA 2130745	A1 19930902	CA 2130745 US 83914492 US 87434492 US 9301576	A 19930222 A 19920220 A 19920424 W 19930222
<input type="checkbox"/> DE 69329154	D1 20000907	DE 69329154 US 9301576 US 83914492 US 87434492	A 19930222 W 19930222 A 19920220 A 19920424
<input type="checkbox"/> DE 69329154	T2 20010607	DE 69329154 US 9301576 US 83914492 US 87434492	T 19930222 W 19930222 A 19920220 A 19920424
<input type="checkbox"/> DE 69333358	D1 20040122	DE 69333358 US 87434492 US 83914492	A 19930222 A 19920424 A 19920220
<input type="checkbox"/> DK 672162	T3 20001218	DK 93906156 US 9301576 US 83914492 US 87434492	T 19930222 W 19930222 A 19920220 A 19920424
		DK 99202507	T 19930222

THIS PAGE IS
INTENTIONALLY
BLANK

<input type="checkbox"/> DK 960944	T3 20040405	DK 93906156	T 19930222
		US 83914492	A 19920220
		US 87434492	A 19920424
<input type="checkbox"/> EP 0672162	A1 19950920	EP 93906156	A 19930222
		US 9301576	W 19930222
		US 83914492	A 19920220
		US 87434492	A 19920424
<input type="checkbox"/> EP 0672162	A4 19951129	EP 93906156	A 19930222
		US 9301576	W 19930222
		US 83914492	A 19920220
		US 87434492	A 19920424
<input type="checkbox"/> EP 0672162	B1 20000802	EP 93906156	A 19930222
		US 9301576	W 19930222
		US 83914492	A 19920220
		US 87434492	A 19920424
<input type="checkbox"/> EP 0960944	A1 19991201	EP 99202507	A 19930222
		EP 93906156	A 19930222
		US 87434492	A 19920424
		US 83914492	A 19920220
<input type="checkbox"/> EP 0960944	B1 20031210	EP 99202507	A 19930222
		EP 93906156	A 19930222
		US 87434492	A 19920424
		US 83914492	A 19920220
<input type="checkbox"/> EP 1378574	A1 20040107	EP 03021426	A 19930222
		EP 99202507	A 19930222
		EP 93906156	A 19930222
		US 83914492	A 19920220
		US 87434492	A 19920424
<input type="checkbox"/> ES 2150938	T3 20001216	ES 93906156	T 19930222
		US 83914492	A 19920220
		US 87434492	A 19920424
<input type="checkbox"/> GR 3034722	T3 20010131	GR 2000402411	T 20001030
		US 9301576	W 19930222
		US 83914492	A 19920220
		US 87434492	A 19920424
<input type="checkbox"/> HK 1003718	A1 20001124	HK 98102884	A 19980407
		US 9301576	W 19930222
		US 83914492	A 19920220
		US 87434492	A 19920424
		HK 00103123	A 19980407

THIS PAGE IS
INTENTIONALLY
BLANK

<input type="checkbox"/> HK 1024936 A1 20040416	US 87434492 A 19920424 US 83914492 A 19920220
<input type="checkbox"/> HK 1024936 A1 20040416	HK 00103123 A 19980407 US 87434492 A 19920424 US 83914492 A 19920220
<input type="checkbox"/> JP 2004000285 A 20040108	JP 2003318959 A 20030910 US 83914492 A 19920220 US 87434492 A 19920424
<input type="checkbox"/> JP 3513151 B2 20040331	JP 51501693 T 19930222 US 9301576 W 19930222 US 83914492 A 19920220 US 87434492 A 19920424
<input type="checkbox"/> JP 7506721 T 19950727	JP 51501693 T 19930222 US 9301576 W 19930222 US 83914492 A 19920220 US 87434492 A 19920424
<input type="checkbox"/> NZ 249734 A 19960827	NZ 24973493 A 19930222 US 83914492 A 19920220 US 87434492 A 19920424
<input type="checkbox"/> PT 672162 T 20010131	PT 93906156 T 19930222 US 83914492 A 19920220 US 87434492 A 19920424
<input type="checkbox"/> PT 960944 T 20040430	PT 99202507 T 19930222 US 83914492 A 19920220 US 87434492 A 19920424
<input type="checkbox"/> SG 46427 A1 19980220	SG 9604612 A 19930222 US 83914492 A 19920220 US 87434492 A 19920424
<input type="checkbox"/> US 5407816 A 19950418	US 83914492 A 19920220 US 87434492 A 19920424
<input type="checkbox"/> WO 9317121 A1 19930902	US 9301576 W 19930222 US 83914492 A 19920220 US 87434492 A 19920424
30 Publications found.	

Copyright © 2004, MicroPatent, LLC. The contents of this page are the property of MicroPatent, LLC including without limitation all text, html, asp, javascript and xml. All rights herein are reserved to the owner and this page cannot be reproduced without the express permission of the owner.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.